Cell

The Evi5 Oncogene Regulates Cyclin Accumulation by Stabilizing the Anaphase-Promoting Complex Inhibitor Emi1

Adam G. Eldridge,[1] Alexander V. Loktev,[1] David V. Hansen,[1] Emmy W. Verschuren,[1] Julie D.R. Reimann,[1] and Peter K. Jackson[1,*]
[1] Departments of Cancer Biology, Pathology, Microbiology and Immunology, and Biophysics, 300 Pasteur Drive, Stanford University School of Medicine, Stanford, CA 94305, USA
*Contact: pjackson@stanford.edu
DOI 10.1016/j.cell.2005.10.038

SUMMARY

The anaphase-promoting complex/cyclosome (APC/C) inhibitor Emi1 controls progression to S phase and mitosis by stabilizing key APC/C ubiquitination substrates, including cyclin A. Examining Emi1 binding proteins, we identified the Evi5 oncogene as a regulator of Emi1 accumulation. Evi5 antagonizes $SCF^{\beta TrCP}$-dependent Emi1 ubiquitination and destruction by binding to a site adjacent to Emi1's DSGxxS degron and blocking both degron phosphorylation by Polo-like kinases and subsequent βTrCP binding. Thus, Evi5 functions as a stabilizing factor maintaining Emi1 levels in S/G2 phase. Evi5 protein accumulates in early G1 following Plk1 destruction and is degraded in a Plk1- and ubiquitin-dependent manner in early mitosis. Ablation of Evi5 induces precocious degradation of Emi1 by the Plk/$SCF^{\beta TrCP}$ pathway, causing premature APC/C activation; cyclin destruction; cell-cycle arrest; centrosome overduplication; and, finally, mitotic catastrophe. We propose that the balance of Evi5 and Polo-like kinase activities determines the timely accumulation of Emi1 and cyclin, ensuring mitotic fidelity.

INTRODUCTION

Progression through the eukaryotic cell cycle is driven by oscillation of the activity of cyclin-dependent kinases (Cdks) (Murray, 2004). Cdk activity is controlled at many levels, but critically by regulating the stability of cyclin itself. Cyclins A and B are destroyed in mitosis through their ability to serve as substrates of the anaphase-promoting complex/cyclosome (APC/C), a large multisubunit E3 ubiquitin ligase (Harper et al., 2002). Like other E3 ubiquitin ligases, the APC/C selects specific protein substrates and directs the formation of ubiquitin chains on them, marking them for destruction by the 26S proteasome (Jackson et al., 2000). What is generally less clear is the identity and mechanism of various accessory factors (Jackson et al., 2000) that regulate the interaction of E3 enzymes with their targets and thereby link protein destruction to the physiological state of the cell.

The APC/C itself is subject to extensive physiological regulation. Its activation is directed by two positive regulators, Cdc20 and Cdh1 (Harper et al., 2002). Cdc20 is known to activate the APC/C during mitosis, whereas Cdh1 activates the APC/C in late mitosis and G1 (Harper et al., 2002). Full ubiquitination activity of the APC/C in mitosis requires its direct phosphorylation by the cyclin B/Cdc2 kinase (Kraft et al., 2003; Harper et al., 2002), as well as the inactivation of several inhibitory proteins that restrain APC/C activity. The proteins MAD2 and BubR1 and other components of the spindle-assembly checkpoint inhibit the APC/C in complex with Cdc20 until all chromosomes are aligned at the metaphase plate and spindle tension is established, thereby ensuring that all kinetochores are attached to both poles of the mitotic spindle (Lew and Burke, 2003). Emi1, originally isolated and described in our lab, inhibits the APC/C in complex with either Cdc20 or Cdh1 and is required to maintain the stability of APC/C substrates such as cyclins A and B in interphase (Hsu et al., 2002; Reimann et al., 2001a, 2001b). Specifically, Emi1 accumulation in late G1 promotes S phase entry by shutting off APC/C^{Cdh1} activity, thereby driving cyclin A accumulation (Hsu et al., 2002). In this context, Emi1 is a central target of the cyclin D/retinoblastoma/E2F pathway because, even in the absence of E2F activation and with only basal levels of cyclin A transcription, Emi1 overexpression is sufficient to drive S phase by stabilizing cyclin A protein. Through its connection to the cyclin D/Rb/E2F pathway, Emi1 provides a critical link from growth-factor stimulation to the inactivation of the APC/C (first described by Lukas et al., 1999) and progression to mitosis (Hsu et al., 2002).

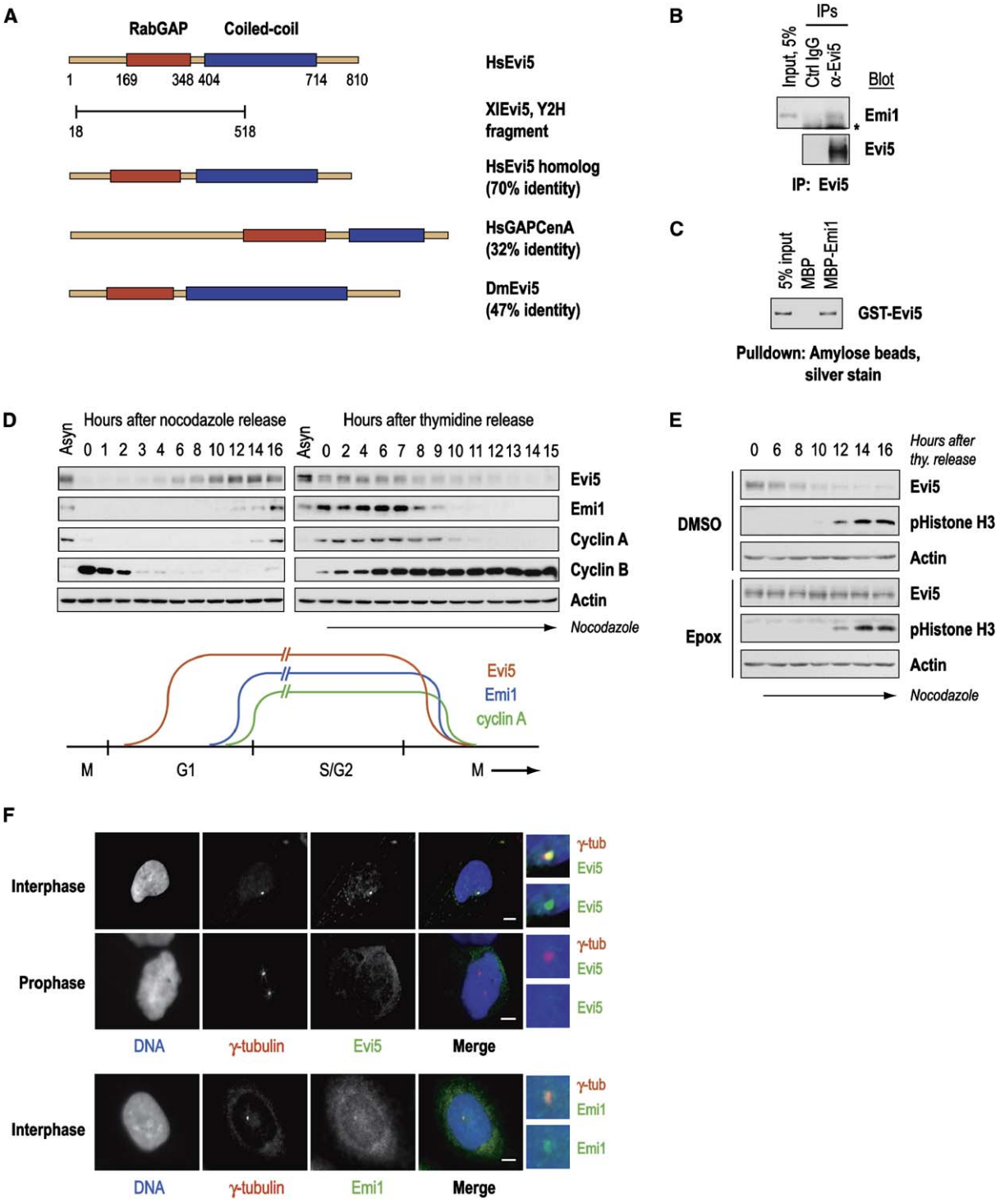


Figure 1. Evi5 Is a Cell-Cycle-Regulated and Centrosomally Localized Emi1-Interacting Protein

(A) Schematic of human Evi5 protein, its closest human homologs, and a candidate Evi5 ortholog in *Drosophila*. The relative positions of the amino-terminal candidate RabGAP domain and carboxy-terminal coiled-coil domain are depicted by red and blue boxes. Mouse and *Xenopus* Evi5 are almost identical to human Evi5 in both domain structure and sequence (91% and 86% identity, respectively).

(B) Endogenous Evi5 and Emi1 proteins form a complex in vivo. Equal amounts of 293T whole-cell lysate were subjected to immunoprecipitation assays using control (normal rabbit IgG) or Evi5 antibodies. Immunoprecipitates were immunoblotted as shown. Asterisk designates crossreactive IgG heavy chain.

Emi1 inactivation in prophase is triggered by its ubiquitination and subsequent degradation by the SCF ubiquitin ligase in complex with the substrate-specific F box adaptor protein βTrCP (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). βTrCP binds a DSGxxS consensus motif in its substrates only when both serines in the motif are phosphorylated (Fuchs et al., 2004). For Emi1, this phosphorylation on the DSGxxS site is triggered in prophase by the Polo-like kinase Plk1 (Hansen et al., 2004; Moshe et al., 2004). $SCF^{\beta TrCP}$ is constitutively active in the cell, and thus the strong activation of Plk1 in mitosis (Golsteyn et al., 1995) would appear to be a sufficient explanation for why Emi1 is stable throughout interphase until cells enter prophase. However, other Polo kinase family members such as Plk2–4, or even low levels of Plk1, may function earlier in the cell cycle, suggesting the need for a mechanism to inhibit Polo-like kinases in interphase (Ang and Harper, 2004).

Although Emi1 functions to stabilize APC/C substrates, little is known about the factors that function upstream of the Emi1 protein to regulate its stability or activity. To better understand Emi1 regulation of the cell cycle, we examined the activities of over 25 *E*mi1-*i*nteracting *p*roteins (EIPs) identified in a well-validated yeast two-hybrid screen for proteins binding to the N-terminal regulatory domain of Emi1 (Reimann et al., 2001a). To look for EIPs that act upstream of Emi1, we undertook a siRNA-based approach to identify factors required for Emi1 stability in vivo. Here, we describe the identification and characterization of the Evi5 oncogene as an EIP required to stabilize the Emi1 protein during interphase. Evi5 was first cloned in mice as a frequent site of retroviral integration in a model of T cell lymphoma (Liao et al., 1997) and later as the site of a reciprocal translocation event in a patient with stage 4S neuroblastoma (Roberts et al., 1998). Though Evi5 contains a conserved amino-terminal Rab GTPase-activating protein homology and a carboxy-terminal coiled-coil domain, little was known about Evi5's physiological role in the cell.

We show here that Evi5 acts to stabilize Emi1 during interphase by blocking the ability of Polo-like kinases to trigger ubiquitin-dependent destruction of Emi1 by the $SCF^{\beta TrCP}$ complex. Notably, the cell-cycle-dependent accumulation of Evi5 itself appears to be largely regulated through its stability and by the activity of Plk1. We find that Evi5 accumulation occurs after Plk1 destruction in G1 and that Evi5 destruction in prophase is triggered by the activation of Plk1. Collectively, our data indicate that Evi5 serves as a critical regulator of cell-cycle progression by defining a window of stability for Emi1 during interphase analogous to the window of stability that Emi1 provides for APC/C substrates over a similar period.

RESULTS

Evi5 Is a Cell-Cycle-Regulated Protein Identified in a Screen for Emi1 Regulatory Proteins

To define Emi1's function in cell-cycle regulation, we had conducted several yeast two-hybrid screens using a *Xenopus* ovary cDNA library and Emi1 amino and carboxyl termini as bait to isolate a number of EIPs (Reimann et al., 2001a). We then screened for EIPs that affected Emi1 accumulation when inactivated by RNAi (A. Lebre, A.G.E., J.D.R.R., and P.K.J., unpublished data). One upstream regulator identified was the *Xenopus* ortholog of Evi5, a protein conserved from humans to flies (Figure 1A). Sequence analysis suggests that Evi5 contains an amino-terminal RabGAP domain (see Figure S1 in the Supplemental Data available with this article online) and a large carboxy-terminal coiled-coil domain. Because of the high degree of identity between *Xenopus* and human Evi5 (86% identity), we chose to characterize human Evi5 in subsequent experiments. Evi5 is a member of a family of homologous proteins exhibiting similar domain structure, including an uncharacterized ORF we call Evi5 homolog (Evi5H) and GAPCenA, a centrosomally localized GTPase-activating protein (GAP) for the Rab6 GTPase (Cuif et al., 1999) (Figure 1A).

To characterize the human Evi5 protein and verify the yeast two-hybrid interaction, we produced affinity-purified rabbit antibodies, which recognized a doublet of species of ~100 kDa on immunoblots of HeLa lysate (Figure S2A). Both species disappeared following blocking of antibody with antigen (Figure S2A) or treatment of cells with siRNA directed against Evi5 but not GFP (Figure S2B), supporting their specificity. Three separate siRNAs targeting the Evi5 mRNA gave identical silencing. Reduced-mobility forms of Evi5 were identified as phosphorylated species by treatment of cell lysates with λ-phosphatase (data not shown). We found that endogenous Emi1 and Evi5 proteins coprecipitate from asynchronous lysates (Figure 1B) and purified,

(C) Recombinant Evi5 binds recombinant Emi1 in vitro. Binding reactions contained each protein at 1 μM final concentration, and MBP-associated complexes were captured on amylose beads, washed, and eluted with SDS sample buffer. Eluates were analyzed by SDS-PAGE and silver staining to detect bound GST-Evi5 protein.

(D) Evi5 protein accumulates in early G1 phase, reaches maximal levels by late G1, and decreases in early mitosis concomitantly with Emi1. HeLa cells were synchronized in mitosis by the nocodazole block-and-release procedure and at the G1/S transition by double-thymidine block followed by release into nocodazole. Time points were analyzed by immunoblot analysis as above.

(E) Evi5 downregulation in early mitosis requires proteasome activity. HeLa cells were synchronized at the G1/S transition by double-thymidine block followed by release into nocodazole. Four hours after release from the second thymidine block, DMSO or the proteasome inhibitor epoxomicin was added to the media. Time points were collected as indicated and analyzed by immunoblotting. Lysates were blotted for phosphorylated histone H3 to verify that treatment with proteasome inhibitor did not block mitotic entry.

(F) Evi5 and Emi1 each localize to the interphase centrosome, but Evi5 leaves the centrosome in prophase. Asynchronous U2OS cells were processed for immunofluorescence analysis using mouse anti-γ-tubulin and rabbit anti-Evi5 or anti-Emi1 antibodies. Cells were fixed with methanol to best preserve centrosomal localization of Emi1. Centrosomes are magnified to the right and are shown with (top) or without (bottom) γ-tubulin staining to emphasize the presence or absence of Evi5 or Emi1 at the centrosome. Bars, 5 μm.

recombinant Emi1 and Evi5 proteins bind in vitro (Figure 1C), providing physical evidence for direct Emi1-Evi5 interaction.

We next asked whether Evi5 protein levels oscillate during the somatic cell cycle. Lysates generated from HeLa cells synchronized by (1) nocodazole block-and-release or (2) double-thymidine block-and-release into nocodazole procedures were analyzed by immunoblotting. Evi5 begins to accumulate in early G1, 6 hr after release from nocodazole, and reaches maximal levels coincident with the beginning of Emi1 accumulation and S phase entry (Figure 1D, left). Blotting lysates from HeLa cells arrested at the G1/S transition by double-thymidine block and then released into nocodazole demonstrated that Evi5 levels drop early in mitosis, at roughly the same time that Emi1 is destroyed (Figure 1D, right). The downregulation of Evi5 in mitosis requires proteasome activity because Evi5 was efficiently stabilized in cells synchronized at the G1/S transition and then released into nocodazole by addition of the specific proteasome inhibitor epoxomicin (Figure 1E).

Examining the subcellular localization of Evi5 by immunofluorescence microscopy, we found that interphase U2OS cells stained with α-Evi5 antibodies showed predominantly centrosomal staining in ~100% of cells, as judged by costaining for γ-tubulin (Figure 1F; see also Faitar et al., 2005). Affinity-purified α-Evi5 antibodies from two other rabbits gave identical staining patterns (data not shown). Blocking the antibodies with antigen (Figure S2C) or inactivation of Evi5 with siRNA (Figure S2D) validated the specificity of Evi5 staining. Notably, Evi5 was absent from the centrosome in mitotic cells (Figure 1F), suggesting that the pool of centrosome-localized Evi5 departs at the same time that the bulk of Evi5 is destroyed in prophase. We also observed staining of Emi1 at interphase centrosomes (Figure 1F), in addition to previous data showing Emi1 in the nucleus (Hsu et al., 2002).

To further test whether Evi5 destruction in prophase requires proteasome-mediated degradation, we showed that (1) the half-life of Evi5 protein in asynchronous HeLa cells is increased from 1 hr to 4 hr by addition of the proteasome inhibitor MG-132 (Figure S3A), (2) the Evi5 protein is ubiquitinated in vivo (Figures S3B and S3C), and (3) mitotic HeLa cell extracts can efficiently ubiquitinate Evi5 protein (Figure S3D). Although Evi5 appears to be destroyed by ubiquitin-dependent proteolysis in early mitosis in mammalian cell culture, Evi5 was not degraded in mitotic or Cdh1-supplemented interphase *Xenopus* egg extracts (Figure S3E), was not ubiquitinated in a standard in vitro APC/C reaction (Figure S3F), and was not stabilized by expression of the APC/C inhibitor Emi1 in asynchronous or mitotic cells (Figure S3G). From these data, we conclude that Evi5 downregulation in early mitosis occurs through a ubiquitin- and proteasome-dependent, but APC/C-independent, mechanism.

Evi5 Contains an Amino-Terminal Emi1 Binding Domain and a Carboxy-Terminal Centrosomal-Targeting Domain

To locate regions of Evi5 required for binding to Emi1 and localization of Evi5, we assayed full-length as well as amino- and carboxy-terminal fragments of Evi5 (Figure 2A) for their ability to bind Emi1 and for their localization in vivo. Coimmunoprecipitation analysis showed that full-length Evi5 bound full-length Emi1 and the Emi1 amino terminus but not the Emi1 carboxyl terminus (Figure 2B, left). Similarly, Emi1 bound the amino terminus of Evi5 efficiently but not the Evi5 carboxyl terminus (Figure 2B, right), showing that each protein binds the other through its amino terminus, consistent with the original yeast two-hybrid data.

As with endogenous Evi5, we found that interphase U2OS cells expressing myc-tagged Evi5 showed prominent centrosomal staining as judged by costaining for γ-tubulin (Figure 2C). myc-Evi5C showed centrosomal staining, whereas myc-Evi5N did not.

We conclude that Evi5 contains two functional domains: an Emi1 binding domain within its N terminus and a centrosomal-localization domain within its C terminus (summarized in Figure 2A). We propose that colocalization of Evi5 with Emi1 at interphase centrosomes may be an important determinant for Evi5's role in stabilizing Emi1 (see below).

The Polo-like Kinase Plk1 Triggers Evi5 Destruction in Mitosis

To understand the cell-cycle regulation of Evi5 levels, we wanted to determine what triggers Evi5 destruction in early mitosis. Phosphorylation by mitotic kinases, including cyclin B/Cdc2 and the Polo-like kinases, is instrumental in effecting critical mitotic transitions. Studies from our lab and others (Hansen et al., 2004; Moshe et al., 2004) showed that Plk1 triggers mitotic destruction of Emi1, and we suspected that Evi5 destruction might be coordinately regulated by Plk1.

Immunoblotting of synchronized lysates (identical to those in Figure 1D) showed that the timing of Plk1 destruction after mitotic exit corresponds closely to the accumulation of Evi5 in early G1, whereas the activation of Plk1 in early mitosis is coincident with the timing of Evi5 destruction (Figure 3A). Moreover, activated Plk1 becomes enriched at centrosomes in early mitosis at roughly the time when Evi5 disappears from centrosomes (Figure 3B). This would be consistent with a model in which high Plk1 kinase activity is antagonistic to Evi5 accumulation or, more directly, phosphorylates Evi5 in mitosis to trigger its destruction. To directly test the role of Plk1 in Evi5 degradation, we immunoblotted lysates generated from HeLa cells treated with GFP or Plk1 siRNA and synchronized by double-thymidine block-and-release into nocodazole (see Figure 2B in Hansen et al., 2004). Although Plk1 siRNA-treated cells showed a 2 hr delay in mitotic entry (as judged by Cdc2 tyrosine dephosphorylation and Cdc27 phosphorylation), Evi5 was significantly stabilized in the absence of Plk1, even at late time points when the Plk1-depleted cells had clearly become mitotic (Figure 3C).

Because Plk1 is required for Evi5 destruction, we asked whether Evi5 was a direct target of Plk1. Evi5 contains six consensus sites (D/E-X-S/T) for phosphorylation by Plk1 (Nakajima et al., 2003) (Figure 3D). To test whether Evi5 is a substrate of Plk1, and to map its phosphorylation sites, we phosphorylated GST-tagged versions of Evi5 in vitro using recombinant *Xenopus* Polo-like kinase (Plx1) (Figure 3E). Full-length Evi5 was efficiently phosphorylated by Plx1, as

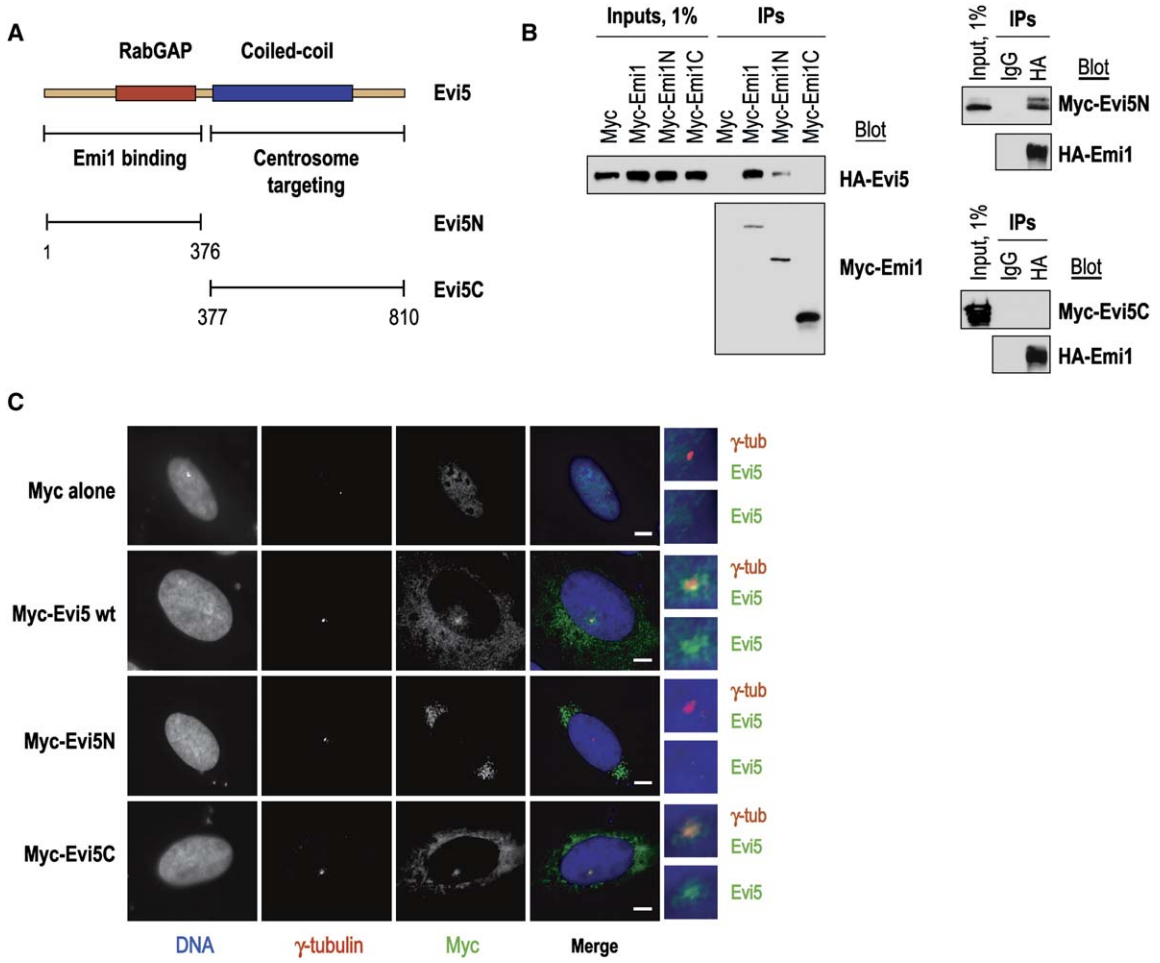


Figure 2. Evi5 Contains an Amino-Terminal Emi1 Binding Domain and a Carboxy-Terminal Targeting Domain

(A) Schematic of full-length human Evi5 protein and fragments used in this study.

(B) Expressed Evi5 binds to Emi1, and each protein binds the other through its amino-terminal domain. Human 293T cells were transfected with expression vectors as indicated. After 48 hr, lysates were generated and normalized, and equal amounts of lysate were used in each binding assay.

(C) Expressed Evi5 is targeted to the centrosome by its carboxy-terminal coiled-coil domain. U2OS cells were transiently transfected with expression vectors as indicated for 48 hr, fixed, and stained with anti-myc antibodies. Centrosomes are magnified to the right and shown with (top) or without (bottom) γ-tubulin staining to emphasize the presence or absence of Evi5 at the centrosome. Bars, 5 μm.

were two individual point mutants in the two amino-terminal Plk1 sites. Conversely, a small deletion of the carboxy-terminal 76 amino acids (ΔC76), which removes the other four candidate Plk1 sites, is a very poor substrate for Plx1 in vitro. An Evi5 mutant lacking all six Plk1 sites was not more compromised in its phosphorylation by Plx1 than the ΔC76 mutant alone was (data not shown), supporting the hypothesis that the C-terminal sites are the most likely sites for Plk1 phosphorylation. Finally, a point mutant missing each of these four carboxy-terminal sites (4×Plk1$^-$) was also fully deficient in phosphorylation by Plx1. Coimmunoprecipitation experiments show that Evi5 can bind directly to Plk1, even in the absence of Plk1 kinase activity (Figure S4; see Discussion).

To determine whether phosphorylation by Plk1 in mitosis is required for Evi5 destruction, we tested the stability of Evi5 wild-type and Plk1-site mutant proteins in mitosis. Expressed wild-type Evi5 protein was efficiently degraded in mitotic HeLa cells, whereas Evi5 ΔC76 and Evi5 4×Plk1$^-$ were substantially stabilized (Figure 3F). Expression of these stabilized Evi5 mutants did not fully stabilize the bulk pool of Emi1 or that of downstream targets of APC/C inhibition by Emi1, such as cyclin A, in these cells. We suspect this may be due to the presence of a second mechanism that inactivates Evi5 in mitosis (see Discussion).

Evi5 Regulates the Accumulation of Emi1 and Cyclin A Proteins in Late G1

To further define the functional relationship between Emi1 and Evi5, we treated cells with siRNA against Evi5 and examined the effect on Emi1 levels. Depletion of Evi5 protein in asynchronously growing HeLa cells results in loss of Emi1, as well as cyclin A and B proteins (Figure S5A). Conversely,

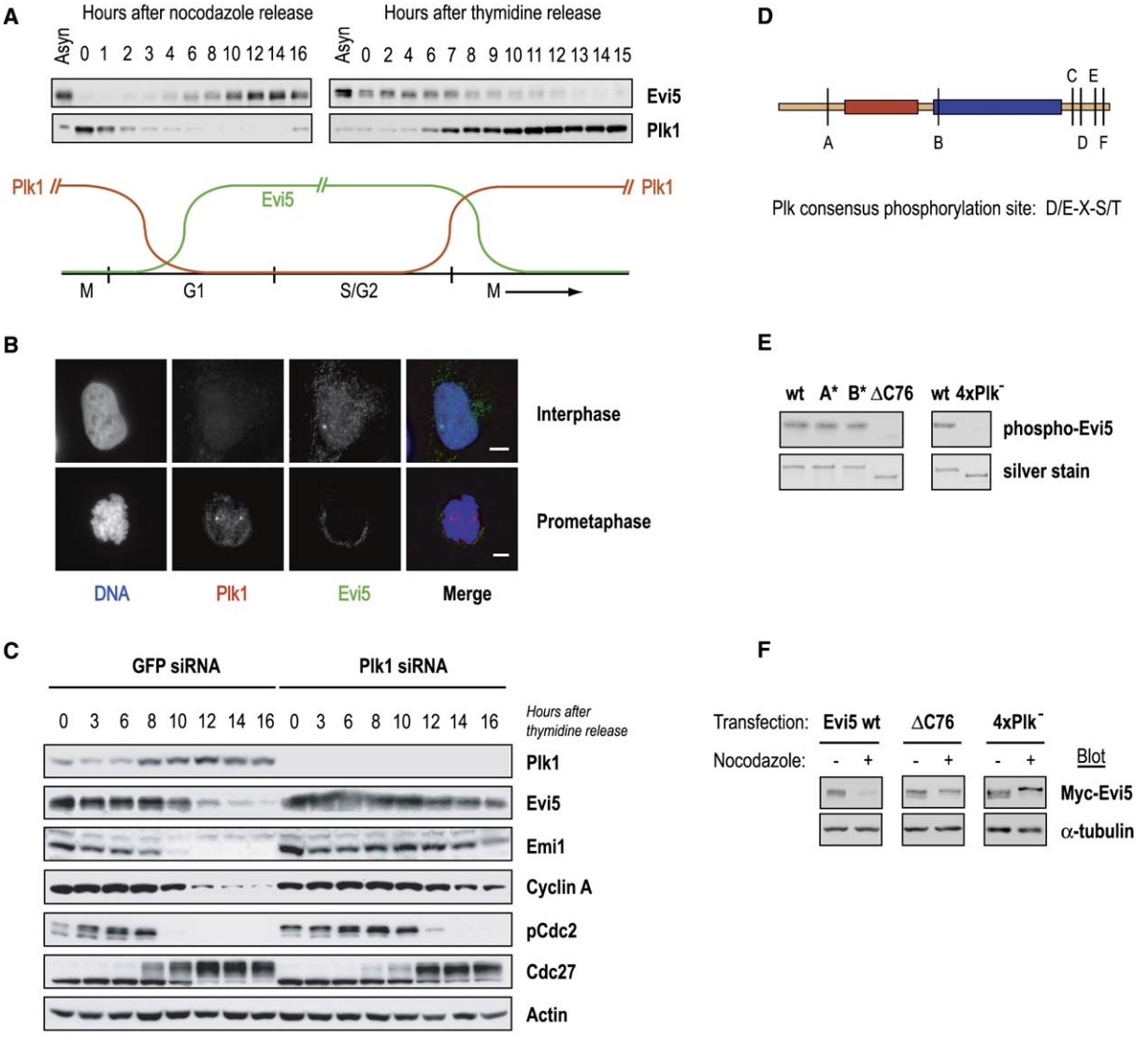


Figure 3. Evi5 Degradation in Early Mitosis Requires Phosphorylation by the Polo-like Kinase Plk1

(A) Cell-cycle oscillation of Evi5 protein levels correlates inversely with Plk1 oscillation. Lysates identical to those used in Figure 1D were analyzed by immunoblot analysis using Evi5 and Plk1 antibodies.

(B) Evi5 localization at the centrosome correlates inversely with presence of Plk1 at the centrosome. Asynchronous U2OS cells were processed for immunofluorescence using anti-Evi5 and anti-Plk1 antibodies, as well as Hoechst to mark DNA. Bars, 5 μm.

(C) Inactivation of Plk1 by RNA interference stabilizes Evi5 in mitosis. HeLa cells were synchronized by double-thymidine block-and-release into nocodazole. siRNA treatment was initiated 4 hr after the release from the first thymidine block. Cells were harvested at the indicated times following release from the second thymidine block, and lysates were generated for immunoblot analysis.

(D) Schematic of candidate Plk1 phosphorylation sites in Evi5. The relative positions of six candidate Plk1 phosphorylation sites (labeled A–F) are shown.

(E) Wild-type Evi5, but not mutants lacking the carboxy-terminal candidate Plk1 sites, is efficiently phosphorylated by Polo-like kinase in vitro. Equal amounts of recombinant wild-type Evi5 as well as mutants lacking candidate Plk1 sites were each phosphorylated in vitro with recombinant Plx1. Reaction products were analyzed by SDS-PAGE and autoradiography as well as silver staining to verify equal loading of Evi5 substrate in each reaction. The 4×Plk1^{-} point mutant has slightly increased mobility, both in vivo and in vitro. Careful sequencing of this mutant eliminated the possibility of premature truncation, and we suspect that its altered mobility is due to deletion of acidic residues within the four Plk1 sites.

(F) Evi5 mutants defective in Plk1 phosphorylation are stabilized in mitosis compared to wild-type protein. Constructs expressing wild-type, ΔC76, or 4×Plk1^{-} versions of Evi5 were transfected into HeLa cells, which were then synchronized in mitosis by treatment with nocodazole. Mitotic cells were harvested by shakeoff, and lysates were generated for immunoblotting.

treatment of HeLa cells with siRNA directed against Emi1 has little effect on Evi5 accumulation but causes significant downregulation of cyclins A and B, both known downstream targets of Emi1 (Figure S5B).

To determine whether the loss of Emi1 and cyclin A in Evi5-depleted cells occurred at the level of protein stability, we treated HeLa cells with GFP or Evi5 siRNA and then synchronized the cells by nocodazole block-and-release. Whereas cells treated with GFP siRNA exit mitosis and accumulate Emi1 and cyclin A normally, cells treated with Evi5 siRNA fail to accumulate these proteins (Figure 4A). Addition of proteasome inhibitor 7 hr after nocodazole release rescued accumulation of both Emi1 and cyclin A (Figure 4A). Thus, the loss of Emi1 and cyclin A protein in cells depleted of Evi5 likely occurs predominantly at the level of protein stability (we exclude checkpoint arrest below). A similar, reciprocal experiment showed that the kinetics of Evi5 accumulation is unchanged in the absence of Emi1, demonstrating that Emi1 is not required to maintain the stability of Evi5 (Figure 4B). To show that Evi5 knockdown did not cause a failure of cells to exit the previous mitosis, we examined early time points after release. We found a slight delay (~2 hr) in mitotic exit as judged by cyclin B degradation and flow cytometry (Figure S6A), but this kinetic effect was too minor to explain the dramatic failure to accumulate Emi1 and cyclin A.

Our earlier work demonstrated that cyclin A accumulation requires inhibition of the APC/C by Emi1 (Hsu et al., 2002). To determine whether Evi5 regulates cyclin A levels indirectly through Emi1 stabilization or by another mechanism, we tested whether we could uncouple cyclin A accumulation from the requirement for Evi5 by expressing a nondegradable Emi1 mutant in Evi5-depleted cells. HeLa cells were transfected with either wild-type or nondegradable Emi1 for 24 hr, followed by the Evi5 depletion and synchronization protocol used in Figure 4A. Expressed wild-type Emi1, like endogenous Emi1, was unstable in the absence of Evi5 protein, whereas nondegradable Emi1 lacking the βTrCP degron remained stable (Figure 4C). Thus, Evi5 directly controls Emi1 protein stability, even when expressed Emi1 is uncoupled from endogenous transcriptional or translational controls. These data suggest that the effect of Evi5 ablation on Emi1 levels is unlikely to arise through secondary effects, such as cell-cycle perturbations, because the transfected Emi1 is constitutively expressed from a strong promoter. Flow cytometry confirmed that HeLa cells depleted of Evi5 by RNAi showed no substantial change in cell-cycle profile (Figure S11). This result also shows that ablation of Evi5 causes Emi1 degradation by the $SCF^{\beta TrCP}$ pathway. Second, nondegradable Emi1, but not wild-type Emi1, fully rescues cyclin A levels in the absence of Evi5 (Figure 4C), suggesting that Evi5's effect on cyclin A stability occurs indirectly through Emi1 stabilization. Thus, Evi5 stabilizes Emi1, which in turn stabilizes cyclin A.

To directly measure the effect of Evi5 depletion on Emi1 protein stability, HeLa cells were arrested in S phase with uniformly high Emi1 and high Evi5 levels by 21 hr thymidine treatment. The cells were then transfected with GFP or Evi5 siRNA in the continued presence of thymidine for an additional 21 hr, after which the cells were released into fresh medium containing cycloheximide to inhibit new protein synthesis. The stability of Emi1 protein was reduced in the absence of Evi5 (Figure 4D), with a reduction in half-life from 3 hr to 1.5 hr, again suggesting that Evi5 functions to stabilize Emi1 directly at the level of protein stability.

To validate the conclusion that Emi1 instability in the absence of Evi5 occurs by the known $SCF^{\beta TrCP}$- (Margottin-Goguet et al., 2003) and Plk1-dependent mechanism (Hansen et al., 2004), we expressed dominant-negative versions of βTrCP and Polo-like kinases and assayed Emi1 levels following Evi5 depletion by RNA interference. Expression of dominant-negative βTrCP (βTrCP ΔF) resulted in substantial stabilization of cotransfected Emi1, nearly to the levels seen in control cells (Figure 4E). Similarly, expression of kinase-defective (kd), dominant-negative versions of Plk1, 2, and 3 in Evi5-depleted cells rescued Emi1 levels (Figure 4F), although to varying extents. The Plk2 rescue vector was most effective, showing a high efficiency of rescue (compared to Plk3-kd) despite its low level of expression (compared to Plk1-kd). We suspect that Plk2-kd, unlike the other Polo-like kinases, rescues efficiently due to its centrosomal localization in interphase (Warnke et al., 2004), where Evi5 is also localized and appears to stabilize Emi1. Together, these results support a model in which Evi5 knockdown by RNA interference precociously activates Plk- and $SCF^{\beta TrCP}$-dependent destruction of Emi1.

Given the centrosomal localization of Evi5, we considered the possibility that Emi1 failed to accumulate in cells lacking Evi5 due to activation of the recently characterized p53-dependent G1 centrosome checkpoint (Borel et al., 2002). We consider this unlikely because HeLa cells are functionally inactive for p53, and direct analysis in matched wild-type or $p53^{-/-}$ HCT116 cells demonstrated that the Evi5 requirement for Emi1 and cyclin stability is independent of p53 (Figure S6B).

We also examined whether Evi5 might affect Emi1 and/or cyclin accumulation by affecting their mRNA levels, which increase in late G1 in response to the E2F transcription factor (Hsu et al., 2002). Examination of mRNA levels by Northern blot analysis of synchronized cells showed that Evi5 mRNA levels do not oscillate in the cell cycle (Figure S7A), in agreement with previously published data (Whitfield et al., 2002). Although Evi5 siRNA treatment followed by nocodazole block-and-release does result in a modest change in Emi1 and cyclin A mRNA levels (Figure S7B), this is unlikely to account for the strong change seen in protein accumulation. Thus, we conclude that Evi5 primarily directs Emi1 and cyclin A accumulation through a strong protein stabilization effect.

Evi5 Maintains Emi1 Stability by Blocking Both Phosphorylation of Emi1 by Polo-like Kinase and Binding of Phosphorylated Emi1 to $SCF^{\beta TrCP}$

We next considered the possibility that Evi5 might stabilize Emi1 in interphase by shielding Emi1 from phosphorylation by Plks. We first tested this hypothesis by determining whether overexpression of Evi5 could block Plk-induced destruction of Emi1 in vivo. Overexpression of Plk1 efficiently

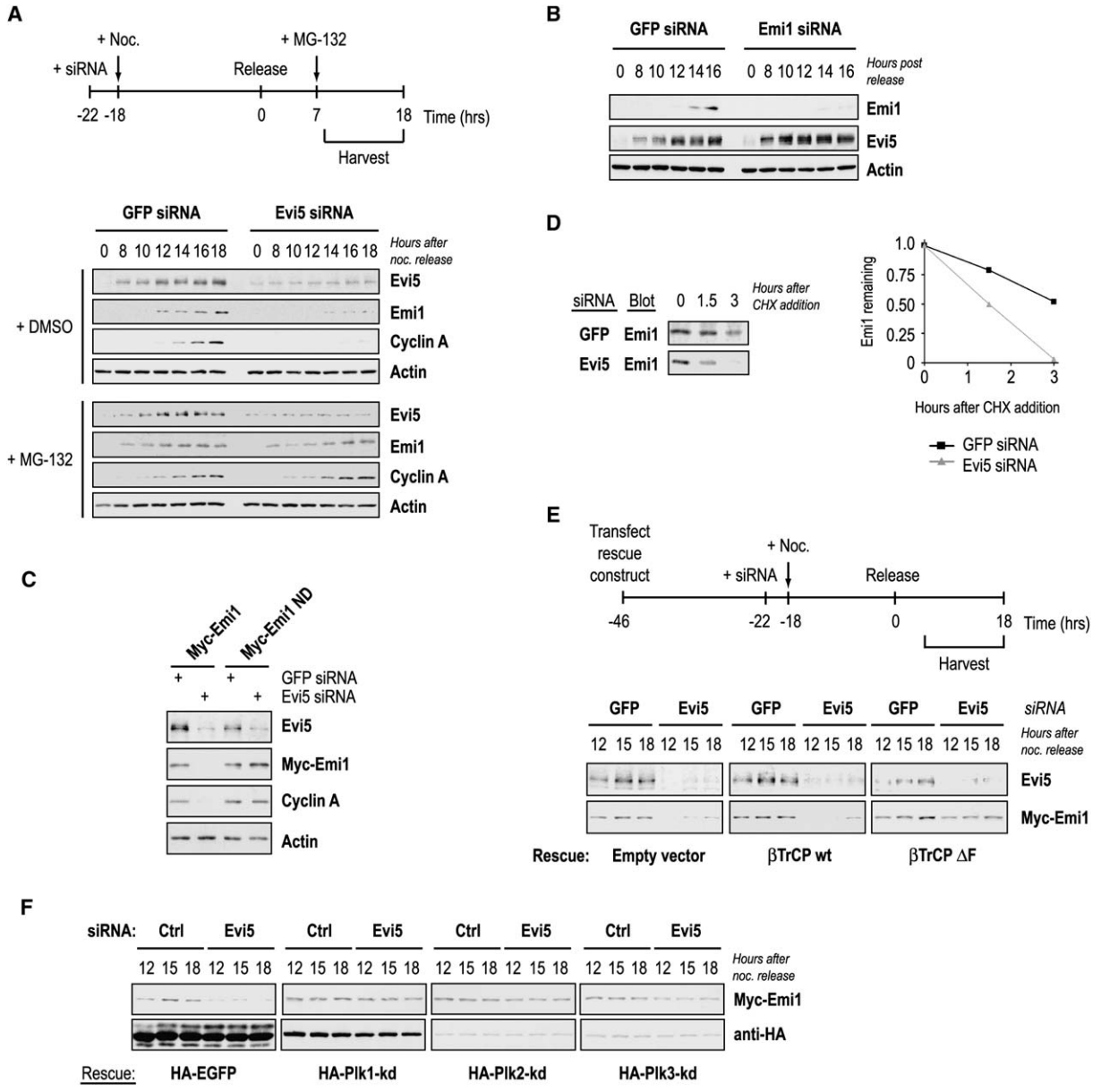


Figure 4. Evi5 Regulates the Stabilization of Emi1 and Cyclin A Proteins in Late G1

(A) Ablation of Evi5 causes failure to accumulate Emi1 and cyclin A proteins in late G1 through a process requiring proteasome activity. HeLa cells were treated with GFP or Evi5 siRNA at t = −22 hr and nocodazole at t = −18 and were released into fresh media at t = 0. Seven hours after nocodazole release, DMSO (above) or proteasome inhibitor MG-132 (below) was added to the media. Time points were collected as indicated and analyzed by immunoblotting.

(B) Emi1 is not required for the accumulation of Evi5 protein in G1. Synchronization of HeLa cells was carried out as in (A), except that cells were treated with siRNA against Emi1 rather than Evi5.

(C) Overexpression of nondegradable Emi1 rescues cyclin A accumulation in Evi5-depleted cells. HeLa cells were transfected with constructs expressing myc-tagged wild-type Emi1 or nondegradable (ND) Emi1 (S145A/S149A lacking the DSGxxS motif required for $SCF^{\beta TrCP}$ binding) for 24 hr and then transfected with siRNA and synchronized with nocodazole as in (A). Cells were harvested 18 hr after release from nocodazole and processed for immunoblot analysis.

(D) Emi1 protein stability is decreased in the absence of Evi5. HeLa cells were arrested in S phase by a single 21 hr thymidine block and then treated with GFP or Evi5 siRNA in the continued presence of thymidine for an additional 21 hr. At time zero, cells were released into normal media and cycloheximide was added to block protein synthesis. Time points were collected, and lysates were analyzed by immunoblotting for Emi1 levels (left) and quantified by densitometry (right).

(E) Downregulation of Emi1 after Evi5 knockdown uses the known βTrCP-dependent pathway for Emi1 destruction. Experiment was as in (C), except that cells were transfected with empty vector, βTrCP wild-type, or dominant-negative βTrCP ΔF constructs in addition to the myc-Emi1 reporter construct.

(F) The destruction of Emi1 induced by Evi5 knockdown requires Polo-like kinase activity. Experiment was as in (E), except that cells were transfected with kinase-defective versions of Plk1, 2, or 3.

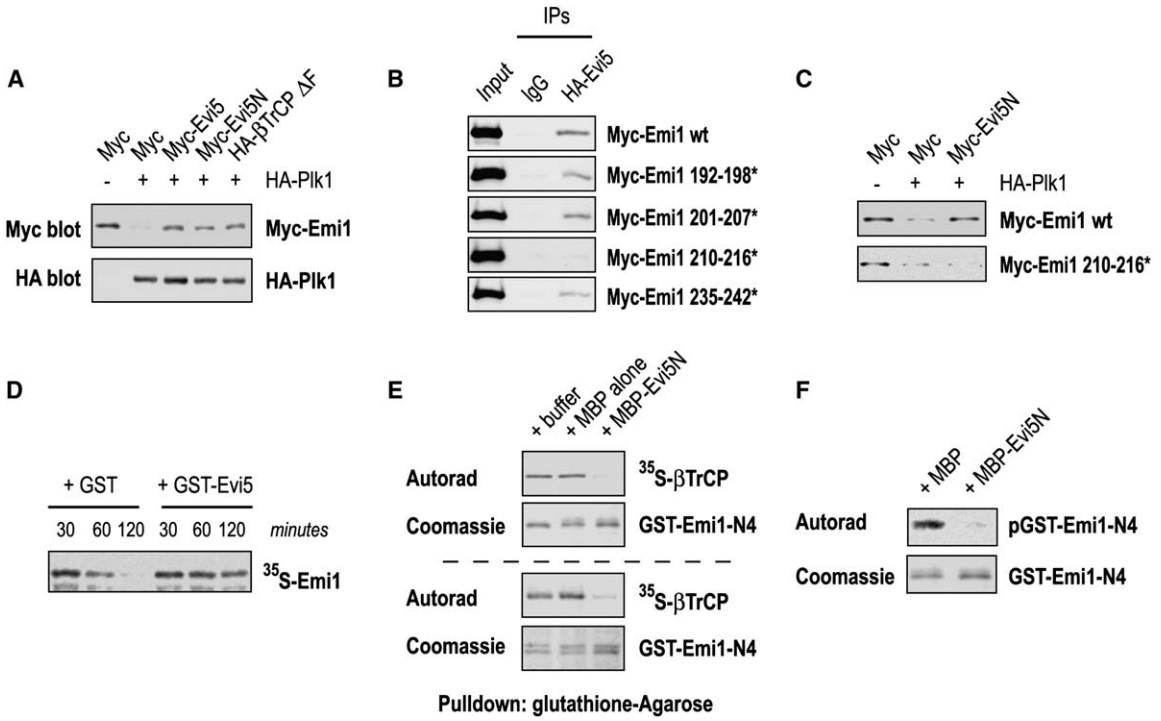


Figure 5. Evi5 Controls Emi1 Stability by Blocking Phosphorylation of the Emi1 Degron by Polo-like Kinase and Recruitment of $SCF^{\beta TrCP}$ to Phosphorylated Emi1

(A) Evi5 overexpression blocks Plk-induced destruction of Emi1 in vivo. 293T cells were cotransfected with myc-Emi1, HA alone, or HA-Plk1, as well as myc alone or myc-Evi5 constructs. After 48 hr, lysates were generated for immunoblotting.

(B) Emi1 requires residues 210–216 to bind Evi5. 293T cells were cotransfected with HA-Evi5 and myc-Emi1 (wild-type or a series of polyalanine point mutants) for 48 hr. Lysates were generated and normalized, and equal amounts were used in each binding assay.

(C) Evi5 does not block Plk-induced destruction of Emi1 210–216*. Assay was conducted as in (A), except that cells were transfected with either Emi1 wild-type or 210–216* vectors.

(D) Evi5 blocks destruction of Emi1 in mitotic HeLa cell lysate. In vitro-translated, ^{35}S-labeled Emi1 was incubated with GST or GST-Evi5 protein, then added to whole-cell lysates generated from HeLa cells synchronized in mitosis by nocodazole block. Time points were removed as indicated and reaction products were analyzed by SDS-PAGE and autoradiography.

(E) Evi5 blocks recruitment of βTrCP to Emi1 in vitro. (Above) GST-Emi1-N4 protein was allowed to bind an excess of MBP or MBP-Evi5N protein, and Emi1 bound complexes were captured on glutathione agarose beads, washed, phosphorylated using recombinant Plx1 kinase, and then incubated with in vitro-translated ^{35}S-labeled βTrCP. Beads were washed and eluted with sample buffer, and eluates were analyzed by SDS-PAGE and autoradiography as well as Coomassie staining to verify equal amounts of Emi1 substrate on the beads in each reaction. (Below) As above, except that GST-Emi1-N4 was first phosphorylated with Plx1 and was then bound to MBP or MBP-Evi5N protein, captured on glutathione agarose beads, and incubated with ^{35}S-labeled βTrCP.

(F) Evi5 blocks phosphorylation of Emi1 by Polo-like kinase in vitro. Experiment was as in (E), except that Emi1-N4 protein was preincubated with MBP or MBP-Evi5N, captured on beads, and phosphorylated using recombinant Plx1 kinase and [γ-^{32}P]ATP. Phosphorylated Emi1 was analyzed by SDS-PAGE and autoradiography as well as Coomassie staining to verify equal amounts of Emi1 substrate on the beads in each reaction.

triggers Emi1 destruction in a proteasome- and $SCF^{\beta TrCP}$-dependent manner (Hansen et al., 2004). Coexpression of Evi5 rescues Emi1 accumulation almost completely, at least as well as expression of dominant-negative βTrCP ΔF (Figure 5A). Expression of Evi5N, which is itself a poor Plk substrate, rescued Emi1 levels as well, suggesting that the rescue phenotype was not merely due to titration of Plk1 kinase activity but rather required the specific N-terminal Emi1 binding function.

To further demonstrate that rescue of Emi1 stability by Evi5 was direct, we first isolated an Emi1 mutant that is unable to bind Evi5 in vivo and then (see below) tested whether this Emi1 mutant was resistant to rescue by Evi5. In vitro binding experiments using deletion fragments of Emi1 showed that Evi5 binds a 110 amino acid region of Emi1 (aa 135–244, Emi1-N4) previously shown to contain the critical phosphodegron for recognition of Emi1 by $SCF^{\beta TrCP}$ and to be fully sufficient for βTrCP binding (Margottin-Goguet et al., 2003) (Figure S8, lanes 1–3). Whereas phosphorylation of both serines within the DSGxxS degron is required for Emi1 binding to βTrCP, mutation of these serines had little effect on binding to Evi5 (Figure S8, lane 4), and prephosphorylation of this motif by Plk1 had no effect on Evi5 binding (data not shown). Thus, Evi5 binds a neighboring but distinct region of Emi1 from the DSGxxS bound by $SCF^{\beta TrCP}$. With this knowledge, we created point mutants in full-length Emi1, converting short patches of conserved residues within the Emi1-N4 region to polyalanine. Coimmunoprecipitation

analysis showed that mutation of residues 210–216 in Emi1 abrogates binding to Evi5 in vivo (Figure 5B). Whereas Emi1 210–216* is degraded like wild-type Emi1 upon Plk1 overexpression, its stability is not rescued by coexpression of Evi5 (Figure 5C). These data support a model in which Evi5 stabilizes Emi1 by directly binding Emi1 and blocking its phosphorylation by Plk1.

To further validate this idea, we turned to in vitro systems. Addition of Evi5 to Emi1 destruction assays carried out in mitotic HeLa cell lysate caused substantial stabilization of the Emi1 substrate (Figure 5D). Incubation of the minimal degron-containing Emi1-N4 fragment with Evi5 caused a marked decline in Plx1-induced binding of Emi1 to βTrCP, either when Evi5 was preincubated with Emi1 prior to Emi1' s phosphorylation by Plk1 (Figure 5E, top) or when Evi5 was incubated with prephosphorylated Emi1 (Figure 5E, bottom). We demonstrated that Evi5 did not bind to βTrCP, excluding the possibility that Evi5 blocked binding of βTrCP to Emi1 by binding βTrCP itself (Figure S9). Finally, preincubation of Emi1 protein with Evi5 resulted in a substantial decrease in Emi1 phosphorylation by Polo kinase (Figure 5F). These data suggest that Evi5 binding is capable of stabilizing Emi1 at two steps: (1) inhibition of phosphorylation by Polo-like kinases and (2) inhibition of recruitment of βTrCP to phosphorylated Emi1. We also asked whether Evi5 bound Emi1 remains capable of inhibiting the APC/C. Indeed, we found that Emi1 bound to Evi5 in vitro does retain its ability to bind both Cdc20 and Cdh1, suggesting that Evi5-bound Emi1 is functional for APC/C inhibition (Figure S10). In summary, our in vitro studies support the conclusion that Evi5 can bind to determinants surrounding the Plk1 phosphorylation-regulated Emi1 degron and serves as a stabilizing factor for Emi1 by blocking precocious triggering of its phosphorylation and destruction.

Evi5 Ablation Causes Cell-Cycle Arrest and Mitotic Abnormalities Due to Increased Centrosome Number

To determine the biological significance of Evi5 expression, we asked whether Evi5 was important to sustain growth and to maintain mitotic and genomic fidelity. Consistent with our data showing that Evi5 is required for Emi1 and cyclin A stability, treatment of asynchronous human retinal pigment epithelial (RPE) cells with Evi5 siRNA resulted in a substantial decrease in growth rate compared to control (Figure 6A) and a dramatic drop in mitotic index (from 2.2% to 0.19%; Figure 6B). Equivalent treatment of U2OS cells caused a smaller drop in mitotic index (3.1% to 1.5%). Inactivation of Evi5 in HeLa cells did show a substantial decrease in proliferation (data not shown), consistent with their failure to accumulate Emi1 and cyclin, but nonetheless showed no clear change in mitotic index upon treatment with Evi5 siRNA. Despite their different effects on overall proliferation, RPE and U2OS cells are similar to HeLa cells in their dependency on Evi5 to maintain Emi1 and cyclin A stability (data not shown).

Flow cytometry of RPE cells treated with Evi5 siRNA showed a significant decrease in G2/M fraction compared to GFP control, whereas equivalent analysis of HeLa and U2OS cells showed essentially unchanged cell-cycle profiles (Figure S11). These flow cytometry data reinforce the argument that the effect of Evi5 siRNA treatment in HeLa cells on Emi1 and cyclin A accumulation is a direct result of knockdown of Evi5 levels rather than an indirect effect caused by cell-cycle perturbations. We suspect that HeLa cells fail to show a drop in mitotic index after Evi5 knockdown because of checkpoint defects or activated oncogene and cyclin/Cdk activities in this highly transformed cell line. Indeed, a large percentage of HeLa cells treated with Evi5 siRNA showed spindle aberrancies in mitosis, many showing increased centrosome and spindle-pole number (Figures 6B and 6C). The increase in centrosome number likely reflects a failure to separate centrosomes into daughter cells following mitotic catastrophe linked to spindle abnormalities. RPE and U2OS cells also accumulated spindle aberrancies, but at a significantly lower frequency (Figure 6B). As is the case with many other growth-regulatory proteins, we suspect that HeLa cells, which are highly transformed and proliferate more quickly than RPE and U2OS cells, are able to bypass the need for Evi5 in regulating mitotic entry. At the same time, the enhanced proliferation rate of HeLa cells may reveal the requirement for Evi5 in centrosome function, mitotic control, and ultimately genomic stability. Thus, in tumor cells, misregulation of Evi5 may drive oncogenic progression by inducing mitotic defects and genome instability.

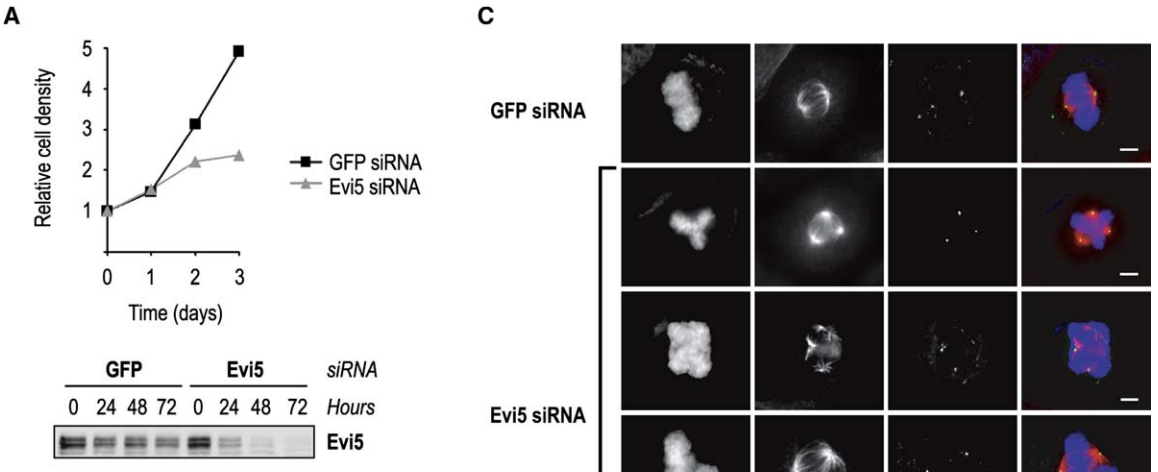
DISCUSSION

We have identified the Evi5 oncogene as a factor critical for the maintenance of Emi1 protein stability and cyclin accumulation during interphase. Evi5 protein accumulates in early G1 and is degraded by ubiquitin-dependent proteolysis in early mitosis in a Plk1-dependent but APC/C-independent manner. Evi5 binding to Emi1 shields Emi1 from phosphorylation by Polo-like kinases and blocks recruitment of phosphorylated Emi1 to $SCF^{\beta TrCP}$ (see Figure 7 for model). Misregulation of Evi5 levels by RNA interference results in cell-cycle arrest and centrosome-number abnormalities in vivo, suggesting that Evi5 may not only regulate cyclin accumulation and cell division but also contribute to timing mechanisms ensuring mitotic fidelity.

The Period of Evi5 Expression during the Cell Cycle Defines a Window of Emi1 and Cyclin Stability

Like cyclin A and Emi1, Evi5 accumulates during G1 and is degraded in early mitosis via ubiquitin-dependent proteolysis. Cyclin A accumulation at the G1/S transition requires both transcription by the growth-factor-responsive E2F transcription factor and stabilization by Emi1 (Hsu et al., 2002). Emi1 expression at G1/S also requires E2F (Hsu et al., 2002), and our studies here suggest that the presence of Evi5 is also required to stabilize newly transcribed and translated Emi1. Evi5 accumulation itself appears to be largely independent of transcription, functioning mainly at the level of protein stability.

We propose that, following mitotic exit, Plk1 is destroyed and Evi5 stability is restored. As cyclin D- and cyclin



B

Cell line	siRNA	Mitotic index (%)	Centrosome number (%) 2	>2
RPE	GFP	2.2	99.0	1.0
	Evi5	0.19	96.8	3.2
U2OS	GFP	3.1	98.0	2.0
	Evi5	1.5	94.3	5.7
HeLa	GFP	1.5	93.3	6.7
	Evi5	1.5	66.7	33.3

Figure 6. Evi5 Misregulation Causes Defects in Cell-Cycle Progression, as Well as Mitotic Abnormalities

(A) Evi5 is required to maintain normal growth rates. Human retinal pigment epithelial (RPE) cells were plated at equal density, transfected with GFP or Evi5 siRNA, and harvested at the indicated time points for counting as well as immunoblot analysis to monitor progression of Evi5 knockdown.

(B) Ablation of Evi5 by RNA interference results in a decrease in mitotic index and an uncoupling of the cell cycle from the centrosome duplication cycle. RPE, U2OS, and HeLa cells were plated onto coverslips, transfected with GFP or Evi5 siRNA, and stained with antibodies against γ-tubulin and α-tubulin and Hoechst to mark DNA. To determine mitotic index, cells were judged to be in mitosis by presence of a mitotic spindle or condensation of DNA. To determine centrosome number, bodies were classed as centrosomes if they stained positively with γ-tubulin and visibly nucleated microtubules by α-tubulin staining.

(C) Ablation of Evi5 by RNA interference results in a high frequency of centrosome abnormalities in HeLa cells. Asynchronous HeLa cells were treated with GFP or Evi5 siRNA for 48 hr and then processed for immunofluorescence using antibodies against α-tubulin and centrin, as well as Hoechst to mark DNA. Bars, 5 μm.

E-dependent kinases become active and begin to inactivate Rb and allow E2F activation, the transcription of Emi1 and cyclin A follows. In the presence of Evi5, Emi1 accumulates, causing inactivation of the APC/C and allowing cyclin A accumulation and S phase entry. APC/C inactivation also allows the stabilization of Skp2 (Ang and Harper, 2004), the F box adaptor that triggers destruction of p27, thus providing an additional mechanism to enhance activation of cyclin E-dependent kinase and E2F activation. As cells progress to early mitosis, the activation of Plk1 triggers the destruction of Evi5 (by an unknown E3) and Emi1 (by $SCF^{\beta TrCP}$) and activation of the APC/C. Because both Emi1 and Evi5 levels are coordinately controlled by phosphorylation by Plk1, we suggest that Evi5 degradation may be triggered specifically in mitosis by a mitotically activated ubiquitin ligase. Phosphorylation of Evi5 by Plk1 in early mitosis could be the critical signal to recruit this ubiquitin ligase. We are currently investigating the determinants of Evi5 degradation.

While it is clear that Plk1 is important for degradation of Evi5 in mitosis, the inability of stabilized Evi5 mutants to stabilize Emi1 in mitosis suggests that an additional inactivation step, such as phosphorylation of Emi1 or Evi5, may be sufficient to remove Evi5 and render Emi1 accessible to phosphorylation by Plk1 and recognition by $SCF^{\beta TrCP}$. Polo-like kinases are recruited to their substrates via binding to phosphorylated motifs in the substrate (Elia et al., 2003). Studies from our lab have shown that phosphorylation of Emi1 by Plk1 is strongly enhanced by prior phosphorylation of Emi1 by Cdc2 (Hansen et al., 2004), and our in vivo binding experiments between Evi5 and Plk1 suggest that Plk1 binds specifically to the phosphorylated form of Evi5 (Figure S4). Thus, both Emi1 and Evi5 are likely phosphorylated by a mitotically activated kinase (or kinases) prior to binding to and phosphorylation by Plk1. Preliminary data suggest that addition of cyclin B/Cdc2 to Emi1/Evi5 binding assays blocks their binding in vitro (A.G.E. and P.K.J., unpublished data). This is consistent with a model in which phosphorylation of Emi1, or Evi5, in early mitosis by cyclin B/cdc2 may be a critical event which coordinately regulates disruption of the Evi5/Emi1 complex as well as recruitment of Plk1 to these

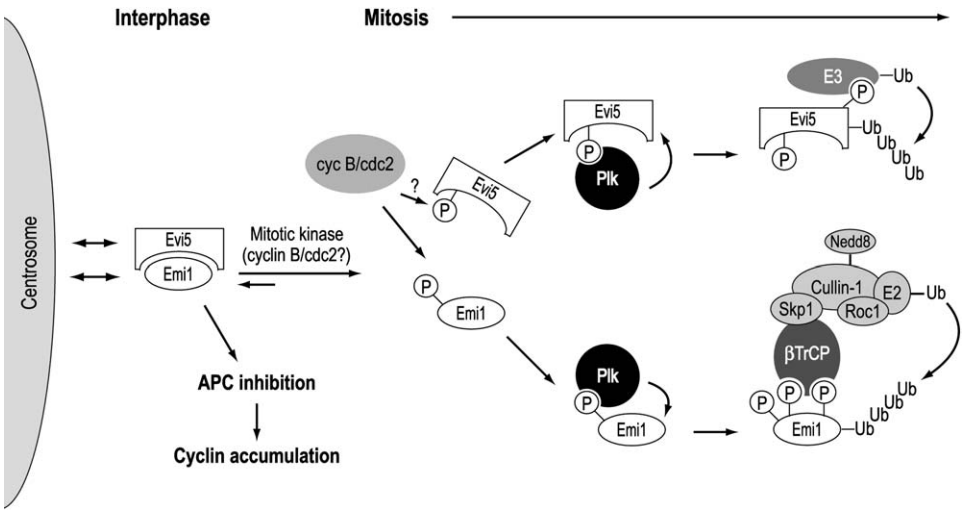


Figure 7. Model

A model is shown depicting stabilization of Emi1 by Evi5 at the centrosome in interphase, as well as the steps in the destruction of both proteins. The ability of Evi5 to stabilize Emi1 may be affected by its association with the centrosome.

proteins. Interestingly, preliminary immunofluorescence data shows that stabilized Evi5 mutants deficient in Plk1 phosphorylation still leave the centrosome in early mitosis when Emi1 becomes concentrated on spindle poles. This change in Evi5 localization in early mitosis, which separates Evi5 and Emi1 spatially, may represent another pathway to inactivate Evi5 in mitosis.

Coupling Evi5 Function at the Centrosome to Emi1 and Cyclin Accumulation

Evi5 localizes to the centrosome from early G1 to prophase. We also find that a pool of Emi1 is centrosomal, suggesting that Evi5 may stabilize Emi1 there (Figure 7). Plk2 activity has been suggested to be required for splitting of centrioles at the G1/S transition (Warnke et al., 2004), and the presence of Evi5 could protect Emi1 from phosphorylation-triggered destruction by Plk2 or another Plk at that time. It should be noted, however, that *Plk2* knockout mice are viable despite displaying some developmental defects, and *Plk2*$^{-/-}$ MEFs are able to grow in culture, albeit at a reduced rate, suggesting that Plk2 is not absolutely required for progression of the centrosome cycle (Ma et al., 2003). Cyclin A, a known APC/C substrate, is required for centriolar splitting at the G1/S transition (Meraldi et al., 1999) and thus might require Evi5 and Emi1 for its stabilization at the centriole. Later, activation of Plk1 may displace Evi5 and Emi1 from the centrosome to destabilize cyclin A and prevent premature splitting of duplicated centrioles in mitosis.

Components of the SCF have also been found at the centrosome (Freed et al., 1999). Notably, βTrCP1 has been found at the centrosome, and knockout of βTrCP1 causes centrosome overduplication in mouse embryo fibroblasts (Guardavaccaro et al., 2003). The SCF component Skp1 and the active, neddylated form of Cul1 are highly enriched at the centrosome, suggesting that an active form of the SCF localizes there (Freed et al., 1999). The possibility of an organized centrosomal ubiquitination/destruction factory has been argued for the spindle-associated destruction of cyclin B (Wakefield et al., 2000) and misfolded proteins in aggresomes (Kopito, 2000). An interesting model posits that Evi5-dependent stabilization of centrosomal Emi1 would primarily direct the accumulation of cyclin A at centrosomes, as suggested above. Another possibility is that there is a critical coupling between the centrosomal and bulk pools of Emi1 and cyclin A, with the centrosomal destruction factory serving to drain the cellular pools of these critical regulators. The ability of Evi5 to "plug" the drain might then couple events at the centrosome to the accumulation of Emi1 and cyclins. Here, Polo-like kinases would provide the critical control to open the drain by triggering Evi5 destruction.

Evi5 Is a Member of a Family of Proteins Containing Amino-Terminal RabGAP Domains and Carboxy-Terminal Coiled-Coil Domains

Evi5 shares a similar RabGAP/coiled-coil domain structure with two other proteins that are conserved from humans to *Drosophila* (see Figure 1A). One of these, GAPCenA, has also been shown to localize to centrosomes through its coiled coil and shows GAP activity for the small GTP binding protein Rab6 in vitro (Cuif et al., 1999). We have not yet shown that Evi5 possesses GAP activity, but the high homology of its GAP domain to those of GAPCenA and other RabGAPs (Figure S1) suggests that it may serve as a GAP for an unknown Rab GTPase. So far, we have not found any phenotypic abnormalities resulting from expression of Evi5 variants containing inactivating point mutations in their GAP domain (A.G.E. and P.K.J., unpublished data). We believe that the GAP function may be distinct from the Emi1 stabilizing

activity and may relate to the interaction of Evi5 with centrosomes or microtubules, possibly as part of a GTPase cycle. An intriguing possibility is that an Evi5-regulated GTPase cycle plays a role in regulating protein stability at the centrosome.

Another homolog, more closely related to Evi5 than GAPCenA, which we call Evi5 homolog (Evi5H), remains as yet completely uncharacterized. Preliminary experiments using RNA interference directed against the uncharacterized Evi5H show a clear difference in function between Evi5 and its homolog; knockdown of the homolog fails to result in the characteristic destabilization of Emi1 and cyclin A seen following Evi5 knockdown but does show other cell-cycle abnormalities (A.G.E. and P.K.J., unpublished data). We are interested in exploring the extent to which these proteins serve similar or differing roles in the cell.

Evi5 Is a Candidate Oncogene that May Regulate Transformation by Perturbing Emi1 and Cyclin Accumulation, Leading to Genomic Instability

The identification of Evi5 as a potential oncogene in mice and humans strongly suggests that Evi5 plays a role in tumorigenesis. We have tested Evi5 for its ability to acutely transform both fibroblasts and interleukin 3-dependent lymphoid (BaF3) cells. To date, expression of Evi5 and all available mutants, including the truncation isolated from a patient with stage 4S neuroblastoma (Roberts et al., 1998), failed to transform cells, suggesting that Evi5 is less likely to be an acutely transforming gene. Rather, we suspect that long-term misregulation of Evi5 in vivo leads to genomic instability, fostering the generation of secondary mutations in the tumorigenic pathway. Misregulation of Emi1 appears to play an important role in driving mitotic catastrophe and genomic instability in a wide variety of human cancers, including many lymphomas, carcinomas, and neural tumors (N. Lehman and P.K.J., unpublished data), and the interaction of Evi5 and Emi1 may point to an involvement of Evi5 in these tumor types.

EXPERIMENTAL PROCEDURES

Cloning and Plasmid and Protein Generation

Xenopus Evi5 sequence was obtained by anchored PCR from a *Xenopus* oocyte cDNA library. Percent identities of Evi5 orthologs and homologs were determined by the Jotun Hein algorithm. Human Evi5, Plk2/3, and variants were cloned into pCS2 myc_6, pCS2 HA_3, pGex 6P-1, or pMAL-c2 as indicated. Emi1 (Hsu et al., 2002), βTrCP (Margottin-Goguet et al., 2003), and Plk1 (Hansen et al., 2004) constructs were previously described. GST and MBP fusions were purified by standard methods. *Xenopus* Plx1 was expressed and purified from baculovirus (Hansen et al., 2004). Cyclin B/Cdc2 kinase was from New England Biolabs. GenBank accession numbers of Evi5 family members are NM_005665 (HsEvi5), AJ011679 (HsGAPCenA), and BC014111 (HsEvi5 homolog).

Plasmid and siRNA Transfections

Plasmid transfections used Fugene 6 (Roche) according to the manufacturer's protocols. siRNA duplexes from Dharmacon were transfected at 50–100 nM final concentration using oligofectamine (Invitrogen).

Antibody Generation

Bacterially produced MBP-Evi5N was used to raise polyclonal antibodies in rabbits (Josman Laboratories). Rabbit sera were subtracted against MBP protein and affinity purified on an MBP-Evi5N column. Monoclonal antibody against human Emi1 (clone 5G12) was from N. Lehman.

Tissue Culture and Immunofluorescence

Cells were fixed in 4% paraformaldehyde and stained as described (Hsu et al., 2002). For centrosomal staining, cells were pre-extracted in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM $MgSO_4$, 0.1% Triton X-100 [pH 7.0]). Microscopy used a Zeiss Axiovert 200M microscope (Intelligent Imaging Innovations) and Slidebook4 software.

Cell Synchronizations and Flow Cytometry

Synchronizations and flow analysis were as described (Hsu et al., 2002). For proteasome inhibition in vivo, MG-132 was used at 20 μM and epoxomicin at 1 μM. For thymidine synchronization followed by cycloheximide treatment, HeLa cells were treated for 21 hr with 2 mM thymidine, transfected with siRNA for 21 hr, and released into fresh media containing cycloheximide (20 μg/ml), and time points were harvested as indicated. Emi1 levels were quantified using ImageQuant software.

Coimmunoprecipitations and Binding Experiments

Coimmunoprecipitations were as described (Hsu et al., 2002), using 300 mM NaCl, 0.3% Triton X-100, 50 mM β-glycerophosphate, 5 mM EDTA, 1 mM DTT, 1% Aprotinin (pH 7.2) as wash buffer. In vitro binding assays were as described (Hansen et al., 2004).

In Vitro Kinase Assays

0.5 μM purified substrate protein was incubated with either 40 ng His-Plx1 or 4 units cyclin B/Cdc2 (New England Biolabs) in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 100 μM cold ATP, and 0.25 μCi/μl [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston). Reactions were incubated at 30ºC for 40 min and analyzed by SDS-PAGE and autoradiography.

Destruction Assays

Destruction assays in HeLa lysate were as described (Montagnoli et al., 1999).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and 11 figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/2/367/DC1/.

ACKNOWLEDGMENTS

We are grateful to J. Cowell for human *Evi5* cDNA; W. Dunphy and A. Kumagai for baculovirus expressing Plx1; W. El-Deiry, R. Erikson, and I. Hoffmann for *Plk2* cDNAs; W. Dai for *Plk3* cDNA; and B. Vogelstein for HCT116 *p53* wild-type and null cells. We thank G. Fang for critical reading of the manuscript and members of the Jackson lab for helpful discussions. This work was funded by grants from the National Institutes of Health to P.K.J. (RO1 GM060439, RO1 GM54811, and RO1 GM73023), a Howard Hughes Predoctoral Fellowship and Stanford Graduate Fellowship to A.G.E., a Damon Runyon Cancer Research Foundation Fellowship (DRG-1811-04) to E.W.V., and a Stanford Graduate Fellowship to D.V.H. This investigation was supported by PHS grant number CA09302, awarded by the National Cancer Institute, DHHS.

Received: May 27, 2005
Revised: August 18, 2005
Accepted: October 21, 2005
Published: January 26, 2006

REFERENCES

Ang, X.L., and Harper, J.W. (2004). Interwoven ubiquitination oscillators and control of cell cycle transitions. Sci. STKE *242*, pe31.

Borel, F., Lohez, O.D., Lacroix, F.B., and Margolis, R.L. (2002). Multiple centrosomes arise from tetraploidy checkpoint failure and mitotic centrosome clusters in p53 and RB pocket protein-compromised cells. Proc. Natl. Acad. Sci. USA *99*, 9819–9824.

Cuif, M.H., Possmayer, F., Zander, H., Bordes, N., Jollivet, F., Couedel-Courteille, A., Janoueix-Lerosey, I., Langsley, G., Bornens, M., and Goud, B. (1999). Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome. EMBO J. *18*, 1772–1782.

Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228–1231.

Faitar, S.L., Dabbeekeh, J.T., Ranalli, T.A., and Cowell, J.K. (2005). EVI5 is a novel centrosomal protein that binds to alpha- and gamma-tubulin. Genomics *86*, 594–605.

Freed, E., Lacey, K.R., Huie, P., Lyapina, S.A., Deshaies, R.J., Stearns, T., and Jackson, P.K. (1999). Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. Genes Dev. *13*, 2242–2257.

Fuchs, S.Y., Spiegelman, V.S., and Kumar, K.G. (2004). The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene *23*, 2028–2036.

Golsteyn, R.M., Mundt, K.E., Fry, A.M., and Nigg, E.A. (1995). Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. J. Cell Biol. *129*, 1617–1628.

Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P.K., Yamasaki, L., and Pagano, M. (2003). Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. Dev. Cell *4*, 799–812.

Hansen, D.V., Loktev, A.V., Ban, K.H., and Jackson, P.K. (2004). Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFbetaTrCP-dependent destruction of the APC Inhibitor Emi1. Mol. Biol. Cell *15*, 5623–5634.

Harper, J.W., Burton, J.L., and Solomon, M.J. (2002). The anaphase-promoting complex: it' s not just for mitosis any more. Genes Dev. *16*, 2179–2206.

Hsu, J.Y., Reimann, J.D., Sorensen, C.S., Lukas, J., and Jackson, P.K. (2002). E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). Nat. Cell Biol. *4*, 358–366.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. *10*, 429–439.

Kopito, R.R. (2000). Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. *10*, 524–530.

Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J.M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. EMBO J. *22*, 6598–6609.

Lew, D.J., and Burke, D.J. (2003). The spindle assembly and spindle position checkpoints. Annu. Rev. Genet. *37*, 251–282.

Liao, X., Du, Y., Morse, H.C., 3rd., Jenkins, N.A., and Copeland, N.G. (1997). Proviral integrations at the Evi5 locus disrupt a novel 90 kDa protein with homology to the Tre2 oncogene and cell-cycle regulatory proteins. Oncogene *14*, 1023–1029.

Lukas, C., Sorensen, C.S., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, J.M., Bartek, J., and Lukas, J. (1999). Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. Nature *401*, 815–818.

Ma, S., Charron, J., and Erikson, R.L. (2003). Role of Plk2 (*Snk*) in mouse development and cell proliferation. Mol. Cell. Biol. *23*, 6936–6943.

Margottin-Goguet, F., Hsu, J.Y., Loktev, A., Hsieh, H.M., Reimann, J.D., and Jackson, P.K. (2003). Prophase destruction of Emi1 by the SCF (betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev. Cell *4*, 813–826.

Meraldi, P., Lukas, J., Fry, A.M., Bartek, J., and Nigg, E.A. (1999). Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. Nat. Cell Biol. *1*, 88–93.

Montagnoli, A., Fiore, F., Eytan, E., Carrano, A.C., Draetta, G.F., Hershko, A., and Pagano, M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. *13*, 1181–1189.

Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor *1*, a regulator of the anaphase promoting complex/cyclosome. Proc. Natl. Acad. Sci. USA *101*, 7937–7942.

Murray, A.W. (2004). Recycling the cell cycle: cyclins revisited. Cell *116*, 221–234.

Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E., and Nishida, E. (2003). Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. J. Biol. Chem. *278*, 25277–25280.

Reimann, J.D., Freed, E., Hsu, J.Y., Kramer, E.R., Peters, J.M., and Jackson, P.K. (2001a). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. Cell *105*, 645–655.

Reimann, J.D., Gardner, B.E., Margottin-Goguet, F., and Jackson, P.K. (2001b). Emi1 regulates the anaphase-promoting complex by a different mechanism than Mad2 proteins. Genes Dev. *15*, 3278–3285.

Roberts, T., Chernova, O., and Cowell, J.K. (1998). NB4S, a member of the TBC1 domain family of genes, is truncated as a result of a constitutional t(1;10)(p22;q21) chromosome translocation in a patient with stage 4S neuroblastoma. Hum. Mol. Genet. *7*, 1169–1178.

Wakefield, J.G., Huang, J.Y., and Raff, J.W. (2000). Centrosomes have a role in regulating the destruction of cyclin B in early Drosophila embryos. Curr. Biol. *10*, 1367–1370.

Warnke, S., Kemmler, S., Hames, R.S., Tsai, H.L., Hoffmann-Rohrer, U., Fry, A.M., and Hoffmann, I. (2004). Polo-like kinase-2 is required for centriole duplication in mammalian cells. Curr. Biol. *14*, 1200–1207.

Whitfield, M.L., Sherlock, G., Saldanha, A.J., Murray, J.I., Ball, C.A., Alexander, K.E., Matese, J.C., Perou, C.M., Hurt, M.M., Brown, P.O., and Botstein, D. (2002). Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol. Biol. Cell *13*, 1977–2000.